

## Rapid Communication

# Hormone Phage: An Enrichment Method for Variant Proteins With Altered Binding Properties

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**ABSTRACT** Human growth hormone (hGH), a 191 residue protein containing two disulfide bonds, was fused to the carboxyl-terminal domain of the gene III protein, a minor coat protein exposed at one end of the filamentous phage M13. The gene fusion was cloned into a plasmid containing origins of replication for *Escherichia coli* and filamentous phage and was packaged into phagemid particles upon infection by an M13KO7 helper phage. Transcription of the hGH-gene III fusion was controlled so that usually no more than one copy of the fusion protein was displayed along with the four copies of the wild-type gene III protein. The hGH-gene III fusion protein was properly folded, as judged by reactivity with six hGH monoclonal antibodies whose epitopes are sensitive to the folded conformation of hGH. Moreover, the hGH-gene III phagemid particles were enriched over 5000-fold from non-hGH phage, and 8-fold from a mutant hGH phagemid following a single hGH-specific elution step from hGH receptor-coated beads. The hGH phagemid should be useful for isolating new receptor binding mutants of hGH. More generally, this expression system may allow other large proteins with discontinuous binding epitopes to be displayed, and binding selections applied to their mutated gene III fusions on filamentous phage.

**Key words:** hormone-receptor interactions, epitope libraries, binding selection, fusion phage, human growth hormone

## INTRODUCTION

Biological selections and screens are powerful tools to probe protein function and to isolate variant proteins with desirable properties. However, a given selection or screen is applicable to only one or a small number of related proteins. Smith and co-workers<sup>1,2</sup> have demonstrated a general method for display of small protein fragments (10–50 amino acids) on the surface of filamentous phage by inserting

short gene fragments into or on the N-terminus of gene III of fd phage ("fusion phage"). The gene III coat protein (present in about five copies at one end of the virion) is important for proper phage assembly and for infection by attachment to the pili of *Escherichia coli* (for review see 3). Recently, fusion phage have been shown to be extremely useful for displaying short mutated peptide sequences (continuous epitopes) for identifying peptides that may react with antibodies<sup>4,5</sup> or a foreign protein.<sup>6</sup>

There are, however, several important limitations in using such fusion phage to identify altered peptides or proteins with new or enhanced binding properties. First, it has been shown<sup>2</sup> that fusion phage are useful only for displaying proteins of less than 100 and preferably less than 50 residues, because large inserts presumably disrupt the function of gene III and therefore phage assembly and infectivity. Second, small peptides generally have a great degree of conformational freedom so that it is extremely difficult to define their binding competent structure, and thereby construct constrained peptide mimics from the sequence of the peptide alone. This would not be as serious a limitation for displaying an epitope library from a large folded protein of known structure. Finally, after exhaustive panning of a random peptide library with an anti- $\beta$ -endorphin monoclonal antibody, Cwirla and co-workers<sup>5</sup> could not separate moderate affinity peptides ( $K_d \sim 10 \mu\text{M}$ ) from higher affinity peptides ( $K_d \sim 0.4 \mu\text{M}$ ) fused to phage. Moreover, the parent  $\beta$ -endorphin

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Abbreviations used: hGH, human growth hormone; hGHbp, human growth hormone binding protein derived from a recombinant fragment corresponding to the extracellular domain of the hGH receptor; Mab, monoclonal antibody; BSA, bovine serum albumin; cfu, colony-forming units; pfu, plaque-forming units.

peptide sequence which is very high affinity ( $K_d \sim 7$  nM) was not panned from the epitope library. These investigators and others<sup>4</sup> have attributed these problems to multiple point attachment of the phage to the solid support due to the presence of five copies of the peptide gene III fusion (a "chelate-effect").

Here we present a potential solution to these problems. We have produced fusion phage which rarely contain more than one copy of the gene III fusion protein per virion particle. This was accomplished by assembling phage particles from a controlled mixture of normal gene III (provided by a helper phage) and a gene III fusion protein under tight transcriptional control (present in a phagemid vector). Such phage are efficiently assembled and propagated without relying on the function of the gene III fusion protein by virtue of their containing at least four copies of the wild-type gene III protein.

## MATERIALS AND METHODS

### Plasmid Constructions and Preparation of hGH-Phagemid Particles

The plasmid pHGH-M13gIII (Fig. 1), was constructed from M13KO7<sup>7</sup> and the hGH producing plasmid, pBO473.<sup>8</sup> A synthetic oligonucleotide 5'-AGC-TGT-GGC-TTC-GGG-CCC-TTA-GCA-TTT-AAT-GCG-GTA-3' was used to produce a unique *Apa*I restriction site (underlined) into pBO473 after the final Phe191 codon of hGH. The oligonucleotide 5'-TTC-ACA-AAC-GAA-GGG-CCC-CTA-ATT-AAA-GCC-AGA-3' was used to introduce a unique *Apa*I restriction site, and a Glu-197-to-amber stop codon (underlined) into M13KO7 gene III. The oligonucleotide 5'-CAA-TAA-TAA-CGG-GCT-AGC-CAA-AAG-AAC-TGG-3' introduces a unique *Nhe*I site (underlined) after the 3' end of the gene III coding sequence. The resulting 650 base pair (bp) *Apa*I-*Nhe*I fragment from the doubly mutated M13KO7 gene III was cloned into the large *Apa*I-*Nhe*I fragment of pBO473 to create the plasmid, pSO132. This fuses the carboxyl terminus of hGH (Phe-191) to the Pro-198 residue of the gene III protein with the insertion of a glycine residue encoded from the *Apa*I site and places the fusion protein under control of the *E. coli* alkaline phosphatase (*phoA*) promoter and *stII* secretion signal sequence.<sup>9</sup> For inducible expression of the fusion protein, we replaced the *phoA* promoter with the *lac* promoter and operator. A 138 bp *Eco*RI-*Xba*I fragment containing the *lac* promoter, operator, and cap binding site was produced by PCR of plasmid pUC119 using the oligonucleotides 5'-CACGACAGAATTCCCGACTGGA-AA-3' and 5'-CTGTTTCTAGAGTGAAATTGTTA-3' that flank the desired *lac* sequences and introduce the *Eco*RI and *Xba*I restriction sites (underlined). This *lac* fragment was gel purified and ligated into the large *Eco*RI-*Xba*I fragment of pSO132 to create the plasmid, pHGH-M13gIII. The sequences of all tailored DNA junctions were verified by the dideoxy

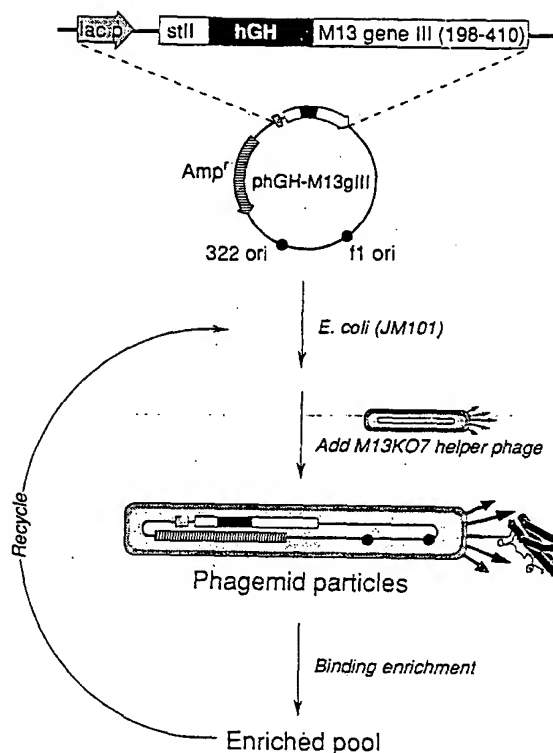


Fig. 1. Strategy for displaying large proteins on the surface of filamentous phage and enriching for altered receptor binding properties. A plasmid, pHGH-M13gIII, was constructed which fuses the entire coding sequence of hGH to the carboxyl-terminal domain of M13 gene III. Transcription of the fusion protein is under control of the *lac* promoter/operator sequence, and secretion is directed by the *stII* signal sequence.<sup>9</sup> Phagemid particles are produced by infection with the "helper" phage, M13KO7,<sup>7</sup> and particles displaying hGH can be enriched by binding to an affinity matrix containing the hGH receptor.<sup>13</sup> The wild-type gene III (derived from the M13KO7 phage) is diagrammed by four to five copies of the multiple arrows on the tip of the phage, and the fusion protein (derived from the phagemid, pHGH-M13gIII) is indicated schematically by the folding diagram of hGH<sup>8</sup> replacing the arrow head. See text for further details.

sequence method.<sup>10</sup> The R64A variant hGH phagemid was constructed as follows: the *Nsi*I-*Bgl*II mutated fragment of hGH<sup>8</sup> encoding the Arg-64 to Ala substitution (R64A)<sup>11</sup> was cloned between the corresponding restriction sites in the pHGH-M13gIII plasmid (Fig. 1) to replace the wild-type hGH sequence. The R64A hGH phagemid particles were propagated and titered as described below for the wild-type hGH phagemid.

Plasmids were transformed into a male strain of *E. coli* (JM101) and selected on carbenicillin plates. A single transformant was grown in 2 ml 2YT medium for 4 hr at 37°C and infected with 50  $\mu$ l of M13KO7 helper phage. The infected culture was diluted into 30 ml 2YT, grown overnight, and phagemid particles were harvested by precipitation with polyethylene glycol.<sup>7</sup> Typical phagemid particle titers ranged from 2 to 5  $\times 10^{11}$  cfu/ml. The

particles were purified to homogeneity by CsCl density centrifugation<sup>12</sup> to remove any fusion protein not attached to virions.

#### Immunochemical Analyses of hGH on the Fusion Phage

Rabbit polyclonal antibodies to hGH were purified with protein A, and coated onto microtiter plates (Nunc) at a concentration of 2 µg/ml in 50 mM sodium carbonate buffer (pH 10) at 4°C for 16–20 hr. After washing in PBS containing 0.05% Tween 20, hGH or hGH phagemid particles were serially diluted from 2.0 to 0.002 nM in buffer A [50 mM Tris (pH 7.5), 50 mM NaCl, 2 mM EDTA, 5 mg/ml bovine serum albumin, and 0.05% Tween 20]. After 2 hr at room temperature (rt), the plates were washed well and the indicated Mab (described in ref. 8) was added at 1 µg/ml in buffer A for 2 hr at rt. Following washing, horseradish peroxidase-conjugated goat anti-mouse IgG antibody was bound at rt for 1 hr. After a final wash, the peroxidase activity was assayed with the substrate, *o*-phenylenediamine.

#### Coupling of the hGH Binding Protein to Polyacrylamide Beads and Binding Enrichments

Oxirane polyacrylamide beads (Sigma) were conjugated to the purified extracellular domain of the hGH receptor (hGHbp)<sup>13</sup> containing an extra cysteine residue introduced by site-directed mutagenesis at position 237 that does not affect binding of hGH (J. Wells, unpublished). The hGHbp was conjugated as recommended by the supplier to a level of 1.7 pmol hGHbp/mg dry oxirane bead, as measured by binding of <sup>125</sup>I-labeled hGH to the resin. Subsequently, any unreacted oxirane groups were blocked with BSA and Tris. As a control for nonspecific binding of phagemid particles, BSA was similarly coupled to the beads. Buffer for adsorption and washing contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 1 mg/ml BSA, and 0.02% Tween 20. Elution buffers contained wash buffer plus 200 nM hGH or 0.2 M glycine (pH 2.1). Parental phage M13KO7 was mixed with hGH phagemid particles at a ratio of nearly 3000:1 (original mixture) and tumbled for 8–12 hr with a 5 µl aliquot (0.2 mg of acrylamide beads) of either absorbent in a 50 µl volume at room temperature. The beads were pelleted by centrifugation and the supernate carefully removed. The beads were resuspended in 200 µl wash buffer and tumbled at room temperature for 4 hr (wash 1). After a second wash (wash 2), the beads were eluted twice with 200 nM hGH for 6–10 hr each (eluate 1, eluate 2). The final elution was with a glycine buffer (pH 2.1) for 4 hr to remove remaining hGH phagemid particles (eluate 3). Each fraction was diluted appropriately in 2YT media, mixed with fresh JM101, incubated at 37°C for 5 min, and plated with

3 ml of 2YT soft agar on LB or LB carbenicillin plates.

## RESULTS AND DISCUSSION

### Construction of hGH Phagemid Particles With a Mixture of Gene III Products

The gene III protein is composed of 410 residues divided into two domains that are separated by a flexible linker sequence.<sup>14</sup> The amino-terminal domain is required for attachment to the pili of *E. coli*, while the carboxyl-terminal domain is imbedded in the phage coat and required for proper phage assembly.<sup>15</sup> The signal sequence and amino-terminal domain of gene III was replaced with the stII signal and entire hGH gene<sup>9</sup> by fusion to residue 198 in the carboxyl-terminal domain of gene III (Fig. 1). The hGH-gene III fusion was placed under control of the *lac* promoter/operator in a plasmid (phGH-M13gIII; Fig. 1) containing the pBR322  $\beta$ -lactamase gene and CoE1 replication origin, and the phage  $\phi$ 1 intergenic region. The vector can be easily maintained as a small plasmid vector by selection on carbenicillin, which avoids relying on a functional gene III fusion for propagation. Alternatively, the plasmid can be efficiently packaged into virions (called phagemid particles) by infection with helper phage such as M13KO7,<sup>7</sup> which avoids problems of phage assembly. Phagemid infectivity titers based upon transduction to carbenicillin resistance in this system varied from 2 to  $5 \times 10^{11}$  colony forming units (cfu)/ml. The titer of the M13KO7 helper phage in these phagemid stocks is  $\sim 10^{10}$  plaque forming units (pfu)/ml.

With this system we found that induction of the *lac* promoter in phGH-M13gIII by addition of IPTG produced low phagemid titers. Moreover, phagemid particles produced by coinfection with M13KO7 containing an amber mutation in gene III gave very low phagemid titers ( $< 10^{10}$  cfu/ml).

It was suggested<sup>4,5</sup> that multiple copies of the gene III fusion can lead to multiple point attachment of the fusion phage to the immobilized target protein. Therefore to control the fusion protein copy number we limited transcription of the hGH-gene III fusion by culturing the plasmid in *E. coli* JM101 (*lacI<sup>R</sup>*) which contains a constitutively high level of the *lac* repressor protein. The *E. coli* JM101 cultures containing phGH-M13gIII were best propagated and infected with M13KO7 in the absence of the *lac* operon inducer (IPTG); however, this system is flexible so that coexpression of other gene III-fusion proteins can be balanced. We estimate that about 10% of the phagemid particles contain one copy of the hGH-gene III fusion protein from the ratio of the amount of hGH per virion (based on hGH immunoreactive material in CsCl gradient purified phagemid). Therefore, the titer of fusion phage dis-

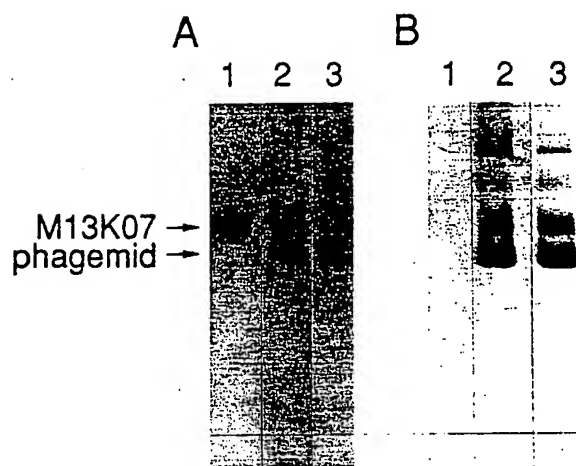


Fig. 2. Immunoblot of whole phage particles shows that hGH comigrates with phage. Phagemid particles purified in a cesium chloride gradient were loaded into duplicate wells and electrophoresed through a 1% agarose gel in 375 mM Tris, 40 mM glycine, pH 9.6, buffer. The gel was soaked in transfer buffer (25 mM Tris, pH 8.3, 200 mM glycine, 20% methanol) containing 2% SDS and 2% 2-mercaptoethanol for 2 hr, then rinsed in transfer buffer for 6 hr. The proteins in the gel were then electroblotted onto immobilon membranes (Millipore). The membrane containing one set of samples was stained with Coomassie blue to show the position of the phage proteins (A). The duplicate membrane was immunostained for hGH by reacting the membrane with polyclonal rabbit anti-hGH antibodies followed by reactions with horseradish peroxidase conjugated goat anti-rabbit IgG antibodies (B). Lane 1 contains the M13K07 parent phage and is visible only in the Coomassie blue-stained membrane, since it lacks hGH. Lanes 2 and 3 contain separate preparations of the hormone phagemid particles which is visible both by Coomassie and hGH immunostaining. The difference in migration distance between the parent M13K07 phage and hormone phagemid particles reflects the different size genomes that are packaged within (8.7 vs. 5.1 kb, respectively).

playing the hGH-gene III fusion is about  $2-5 \times 10^{10}$  ml. This number is much greater than the titer of *E. coli* ( $\sim 10^8$  to  $10^9$ /ml) in the culture from which they are derived.

#### Structural Integrity of the hGH-Gene III Fusion

Immunoblot analysis (Fig. 2) of the hGH-gene III phagemid shows that hGH cross-reactive material comigrates with phagemid particles in agarose gels. This indicates that the hGH is tightly associated with phagemid particles. The hGH-gene III fusion protein from the phagemid particles runs as a single immunostained band showing that there is little degradation of the hGH when it is attached to gene III. Wild-type gene III protein is clearly present because about 25% of the phagemid particles are infectious. This is comparable to specific infectivity estimates made for wild-type M13 phage that are similarly purified (by CsCl density gradients) and concentrations estimated by UV absorbance.<sup>1,2</sup> Thus, both wild-type gene III and the hGH-gene III fusion proteins are displayed in the phage pool.

It was extremely important to confirm that the

TABLE 1. Binding of Eight Different Monoclonal Antibodies (Mab's) to hGH and hGH Phagemid Particles\*

Mab	IC <sub>50</sub> (nM)	
	hGH	hGH phagemid
1	0.4	0.4
2	0.04	0.04
3	0.2	0.2
4	0.1	0.1
5	0.2	>2.0
6	0.07	0.2
7	0.1	0.1
8	0.1	0.1

\*Values given represent the concentration (nM) of hGH or hGH phagemid particles to give half-maximal binding to the particular Mab. Standard errors in these measurements are typically at or below  $\pm 30\%$  of the reported value. See Materials and Methods for further details.

tertiary structure of the displayed hGH was maintained in order to have confidence that results from binding selections will translate to the native protein. We used monoclonal antibodies (Mabs) to hGH to evaluate the structural integrity of the displayed hGH-gene III fusion protein (Table I). The epitopes on hGH for these Mabs have been mapped<sup>8</sup> and binding for seven of eight Mabs requires that hGH be properly folded. The IC<sub>50</sub> values for all Mabs were equivalent to wild-type hGH except for Mab 5 and 6. Both Mabs 5 and 6 are known to have binding determinants near the carboxyl-terminus of hGH which is blocked in the gene III fusion protein. The relative IC<sub>50</sub> value for Mab1 which reacts with both native and denatured hGH is unchanged compared to the conformationally sensitive Mabs 2-5, 7, and 8. Thus, Mab1 serves as a good internal control for any errors in matching the concentration of the hGH standard to that of the hGH-gene III fusion.

#### Binding Enrichments on Receptor Affinity Beads

Previous workers<sup>2,4-6</sup> have fractionated phage by panning with streptavidin-coated polystyrene petri dishes or microtiter plates. However, chromatographic systems would allow more efficient fractionation of phagemid particles displaying mutant proteins with different binding affinities. We chose nonporous oxirane beads (Sigma) to avoid trapping of phagemid particles in the chromatographic resin. Furthermore, these beads have a small particle size (1  $\mu$ m) to maximize the surface area to mass ratio. The extracellular domain of the hGH receptor (hGHbp)<sup>13</sup> containing a free cysteine residue was efficiently coupled to these beads and phagemid particles showed very low non-specific binding to beads coupled only to bovine serum albumin (Table II).

In a typical enrichment experiment (Table II), one part of hGH phagemid was mixed with >3000 parts

TABLE II. Specific Binding of Hormone Phage to hGHbp-Coated Beads Provides an Enrichment for hGH Phage Over M13KO7 Phage\*

Sample	Absorbent <sup>‡</sup>	Total pfu	Total cfu	Ratio (cfu/pfu)	Enrichment <sup>§</sup>
Original mixture <sup>†</sup>		$8.3 \times 10^{11}$	$2.9 \times 10^8$	$3.5 \times 10^{-4}$	(1)
Supernatant	BSA	$7.4 \times 10^{11}$	$2.8 \times 10^8$	$3.8 \times 10^{-4}$	1.1
	hGHbp	$7.6 \times 10^{11}$	$3.3 \times 10^8$	$4.3 \times 10^{-4}$	1.2
Wash 1	BSA	$1.1 \times 10^{10}$	$6.0 \times 10^6$	$5.5 \times 10^{-4}$	1.6
	hGHbp	$1.9 \times 10^{10}$	$1.7 \times 10^7$	$8.9 \times 10^{-4}$	2.5
Wash 2	BSA	$5.9 \times 10^7$	$2.8 \times 10^4$	$4.7 \times 10^{-4}$	1.3
	hGHbp	$4.9 \times 10^7$	$2.7 \times 10^6$	$5.5 \times 10^{-2}$	$1.6 \times 10^2$
Eluate 1 (hGH)	BSA	$1.1 \times 10^6$	$1.9 \times 10^3$	$1.7 \times 10^{-3}$	4.9
	hGHbp	$1.2 \times 10^6$	$2.1 \times 10^6$	1.8	$5.1 \times 10^3$
Eluate 2 (hGH)	BSA	$5.9 \times 10^5$	$1.2 \times 10^3$	$2.0 \times 10^{-3}$	5.7
	hGHbp	$5.5 \times 10^5$	$1.3 \times 10^6$	2.4	$6.9 \times 10^3$
Eluate 3 (pH 2.1)	BSA	$4.6 \times 10^5$	$2.0 \times 10^3$	$4.3 \times 10^{-3}$	12.3
	hGHbp	$3.8 \times 10^5$	$4.0 \times 10^6$	10.5	$3.0 \times 10^4$

\*The titers of M13KO7 and hGH phagemid particles in each fraction was determined by multiplying the number of plaque forming units (pfu) or carbenicillin-resistant colony forming units (cfu) by the dilution factor, respectively. See Materials and Methods for details.

<sup>†</sup>The ratio of M13KO7 to hGH phagemid particles was adjusted to 3000:1 in the original mixture.

<sup>‡</sup>Absorbents were conjugated with BSA or hGHbp.

<sup>§</sup>Enrichments are calculated by dividing the cfu/pfu ratio after each step by cfu/pfu ratio in the original mixture.

M13KO7 phage. After one cycle of binding and elution,  $10^6$  phage were recovered and the ratio of phagemid to M13KO7 phage was 2 to 1. Thus, a single binding selection step gave >5000-fold enrichment. Additional elutions with free hGH or acid treatment to remove remaining phagemids produced even greater enrichments. The enrichments are comparable to those obtained by Smith and co-workers using batch elution from coated polystyrene plates,<sup>1,2</sup> however small volumes are used on the beads (200  $\mu$ l vs. 6 ml). There was almost no enrichment for the hGH phagemid over M13KO7 when we used beads linked only to BSA. The slight enrichment observed for control beads (~10-fold for pH 2.1 elution; Table II) may result from trace contaminants of bovine growth hormone binding protein present in the BSA linked to the bead. Nevertheless these data show the enrichments for the hGH phage depend upon the presence of the hGHbp on the bead suggesting binding occurs by specific interaction between hGH and the hGHbp.

We evaluated the enrichment for wild-type hGH over a weaker binding variant of the hGH on fusion phagemids to further demonstrate enrichment specificity, and to link the reduction in binding affinity for the purified hormones to enrichment factors after panning fusion phagemids. A fusion phagemid was constructed with an hGH mutant in which Arg-64 was substituted with Ala (R64A). The R64A variant hormone is about 20-fold reduced in receptor binding affinity compared to hGH ( $K_d$  values of 7.1 and 0.34 nM, respectively<sup>11</sup>). The titers of the R64A hGH-gene III fusion phagemid were comparable to those of wild-type hGH phagemid. After one round

of binding and elution (Table III) the wild-type hGH phagemid was enriched from a mixture of the two phagemids plus M13KO7 by 8-fold relative to the phagemid R64A, and  $\sim 10^4$  relative to M13KO7 helper phage.

## CONCLUSIONS

By displaying a mixture of wild-type gene III and the gene III fusion protein on phagemid particles one can assemble and propagate virions that display a large and properly folded protein as a fusion to gene III. The copy number of the gene III fusion protein can be effectively controlled to avoid "chelate effects" yet maintained at high enough levels in the phagemid pool to permit panning of large epitope libraries ( $>10^{10}$ ). We have shown that hGH (a 22 kDa protein) can be displayed in its native folded form. Binding selections performed on receptor affinity beads eluted with free hGH efficiently enriched for wild-type hGH phagemids over a mutant hGH phagemid shown to have reduced receptor binding affinity. Thus, it is possible to sort phagemid particles whose binding constants are down in the nanomolar range.

Protein-protein and antibody-antigen interactions are dominated by discontinuous epitopes;<sup>16-19</sup> that is the residues directly involved in binding are close in tertiary structure but separated by residues not involved in binding. The screening system presented here should allow one to analyze more conveniently protein-receptor interactions and isolate discontinuous epitopes in proteins with new and high affinity binding properties.

TABLE III. hGHbp-Coated Beads Select for hGH Phagemids Over a Weaker Binding hGH Variant Phagemid\*

Sample	Control beads		hGHbp beads	
	WT phagemid total phagemid	Enrichment for WT/R64A	WT phagemid total phagemid	Enrichment <sup>†</sup> for WT/R64A
Original mixture	8/20	(1)	8/20	(1)
Supernatant	ND	—	4/10	1.0
Elution 1 (hGH)	7/20	0.8	17/20	8.5 <sup>‡</sup>
Elution 2 (pH 2.1)	11/20	1.8	21/27	5.2

\*The parent M13KO7 phage, wild-type hGH phagemid and R64A phagemid particles were mixed at a ratio of 10<sup>4</sup>:0.4:0.6. Binding selections were carried out using beads linked with BSA (control beads) or with the hGHbp (hGHbp beads) as described in Table II and Materials and Methods. After each step, plasmid DNA was isolated<sup>20</sup> from carbenicillin-resistant colonies and analyzed by restriction analysis to determine if it contained the wild-type hGH or the R64A hGH-gene III fusion.

<sup>†</sup>The enrichment for wild-type hGH phagemid over R64A mutant was calculated from the ratio of hGH phagemid present after each step to that present in the original mixture (8/20), divided by the corresponding ratio for R64A phagemids. WT, wild-type; ND, not determined.

<sup>‡</sup>The enrichment for phagemid over total M13KO7 parental phage was ~10<sup>4</sup> after this step.

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